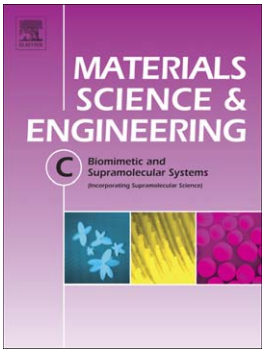


Enzyme mediated synthesis of polypyrrole in the presence of chondroitin sulfate and redox mediators of natural origin

G.A. Grijalva-Bustamante, A.G. Evans-Villegas, T. del Castillo-Castro, M.M. Castillo-Ortega, R. Cruz-Silva, F. Huerta, E. Morallón

PII: S0928-4931(16)30222-3
DOI: doi: 10.1016/j.msec.2016.03.042
Reference: MSC 6307

To appear in: *Materials Science & Engineering C*

Received date: 2 December 2015
Revised date: 2 March 2016
Accepted date: 14 March 2016

Please cite this article as: G.A. Grijalva-Bustamante, A.G. Evans-Villegas, T. del Castillo-Castro, M.M. Castillo-Ortega, R. Cruz-Silva, F. Huerta, E. Morallón, Enzyme mediated synthesis of polypyrrole in the presence of chondroitin sulfate and redox mediators of natural origin, *Materials Science & Engineering C* (2016), doi: 10.1016/j.msec.2016.03.042

Enzyme mediated synthesis of polypyrrole in the presence of chondroitin sulfate and redox mediators of natural origin

G.A. Grijalva-Bustamante [a], A.G. Evans-Villegas [b], T. del Castillo-Castro [a,*], M.M. Castillo-Ortega [a], R. Cruz-Silva [c], F. Huerta [d], E. Morallón [e]

[a] *Departamento de Investigación en Polímeros y Materiales, Universidad de Sonora, CP 83000, Hermosillo, Sonora, Mexico*

[b] *Departamento de Ciencias Químico Biológicas, Universidad de Sonora, CP 83000, Hermosillo, Sonora, Mexico*

[c] *Research Center for Exotic Nanocarbons, Shinshu University, 4-17-1 Wakasato, 380-8553, Nagano, Japan.*

[d] *Departamento Ingeniería Textil y Papelera, Universitat Politecnica de Valencia, Plaza Ferrandiz y Carbonell, 1. E-03801, Alcoy, Spain*

[e] *Departamento Química Física e Instituto Universitario de Materiales, Universidad de Alicante, Ap. 99, E-03080, Alicante, Spain*

* Corresponding author. Tel.: +52 662 2592161; fax: +52 662 2592216

E-mail address: terecat@polimeros.uson.mx (T. del Castillo-Castro).

Abstract

Polypyrrole (PPy) was synthesized by enzyme mediated oxidation of pyrrole using naturally occurring compounds as redox mediators. The catalytic mechanism is an enzymatic cascade reaction in which hydrogen peroxide is the oxidizer and soybean peroxidase, in the presence of acetosyringone, syringaldehyde or vanillin, acts as a natural catalysts. The effect of the initial reaction composition on the polymerization yield and electrical conductivity of PPy was analyzed. Morphology of the PPy particles was studied by scanning electron microscopy and transmission electron microscopy whereas the chemical structure was studied by X-ray photoelectron and Fourier transformed infrared spectroscopic techniques. The redox mediators increased the polymerization yield without a significant modification of the electronic structure of PPy. The highest conductivity of PPy was reached when chondroitin sulfate was used simultaneously as dopant and template during pyrrole polymerization. Electroactive properties of PPy obtained from natural precursors were successfully used in the amperometric quantification of uric acid concentrations. PPy increases the amperometric sensitivity of carbon nanotube screen-printed electrodes toward uric acid detection.

Keywords: *biocatalytic polymerization; polypyrrole, natural redox mediators; uric acid sensor.*

1. Introduction

Polypyrrole (PPy) is one of the most investigated conducting polymers for biomedical applications due to its biocompatibility with several cell types [1,2] and high potential as electroconductive coating of scaffolds for tissue engineering [3].

PPy is frequently synthesized by chemical or electrochemical oxidation methods. Chemical polymerization is often associated with generation of toxic byproducts, it is usually performed in high acidity reaction media, and requires a large amounts of oxidant [4,5]. Similarly, the electrochemical synthesis is carried out under acid conditions, and the polymer deposition is limited to the electrode surface [6,7].

Enzymatic mediated synthesis of PPy is an environmentally friendly approach for producing the polymer under "soft" conditions while avoiding the generation of toxic byproducts. However, polymerization of pyrrole catalyzed by enzymes has received less attention as compared to the well-known enzymatic synthesis of other electrically conductive polymers, such as polyaniline [8,9]. This has been associated to the low catalytic activity of most oxidoreductase enzymes, commonly used for enzyme-catalyzed polymerizations, towards pyrrole. Ramanavicius *et al.* [10] used glucose oxidase as catalyst and found that *in situ* enzymatically generated hydrogen peroxide could be used as oxidizer for the polymerization of pyrrole. Interestingly, the polymerization rate was directly correlated to the catalytic activity of the enzyme.

During the last decade, several methods have overcome the limitations for enzyme-mediated synthesis of PPy by introducing redox mediators that act as electron shuttles, enabling the oxidative polymerization of pyrrole. Song and Palmore [11] reported the laccase-catalyzed polymerization of pyrrole by dioxygen in the presence of 2,2´-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) at pH 4. They found that ABTS behaved both as redox mediator and as dopant during the pyrrole polymerization.

Kupriyanovich *et at.* [12] studied the polymerization of pyrrole by using hydrogen peroxide and horseradish peroxidase (HRP) in the presence of ABTS mediator and electrolytes at pH 4.5. The highest conductivity of polymer was reached when sodium polystyrene-4-sulfonate (PSS) was used as dopant and template to induce the ordered growth of the PPy backbone.

Cruz-Silva *et al.* [13] synthesized PPy in high yield also using the hydrogen peroxide/HRP/ABTS system at pH 4. The same conditions were used to produce films and water-dispersible PPy colloids by adding polyvinyl alcohol as steric stabilizer.

In a different approach, Bouldin *et al.* [14] reported the enzymatic-mediated formation of PPy with linear structure by using hydrogen peroxide/soybean peroxidase (SBP) at pH 3.5 without the aid of a redox mediator. PSS was used as a charge-balancing dopant and dispersant for PPy, achieving conductivities that exceeded 3 S cm^{-1}. Recently, laccase has also been used to synthesize PPy confined within polymer vesicles [15]. The characterization of PPy obtained through enzyme-mediated catalysis has shown that a fraction of the enzymes, redox mediators and dopant-templates remain within the structure of the synthesized polymer. Therefore, the potential toxicity of the reagents involved in the synthesis of PPy should be considered when the polymer is aimed at biomedical applications.

In this work, SBP-mediated synthesis of PPy using acetosyringone (AS), syringaldehyde (SA) or vanillin (VA) as redox mediators is presented. These mediators are naturally-occurring phenols that have been widely used as laccase mediators in different oxidation processes [16]. ABTS was also used as redox mediator for comparison purpose. In order to use mainly bio-based materials, we have chosen two natural polymers, hyaluronic acid (HA) and chondroitin sulfate (CS), which were incorporated during the synthesis as templates for PPy growth and dopant. Both HA and CS are anionic polyelectrolytes with potential use in biomedical field [17]. Taking advantage of the electrochemical activity of the synthesized PPy, we prepared an amperometric sensor for uric acid (UA) detection. The amperometric techniques are powerful analytical tools that are widely used in the electrochemical detection of biologically and clinically important species [18–21].

The aim of this work is to study the possibility of synthesizing electroactive PPy in an environmentally friendly process by using natural polymers and redox mediators obtained from natural renewable resources.

2. Experimental Section

2.1 Chemicals

Soybean peroxidase (SBP; EC 1.11.1.7) was purchased from Bioresearch Products Inc (USA). The activity of enzyme reagent (native SBP) was 1280 ± 86 IU mg^{-1}, determined with guaiacol substrate (Aldrich, China) in buffer of 2-(N-Morpholino)ethanesulfonic acid (MES, 50 Mm, pH 6) (Aldrich, USA). A portion of the enzyme reagent was thermally treated at 100°C for 20 min in aqueous solution (denatured SBP) for control experiment. The residual activity of the denatured SBP was not determined by the standard procedure; it was considered that this thermal treatment is typically enough to reach its total inactivation [22–24]. Pyrrole (Py) (Aldrich, USA) was distilled under vacuum before use and stored in dark at about 0°C. Hydrogen peroxide (H_2O_2, 50%wt) (Aldrich, USA), acetosyringone (AS) (Aldrich, USA), syringaldehyde (SA) (Aldrich, Japan), vanillin (VA) (Aldrich, China), hyaluronic acid (HA) (Aldrich, Germany), chondroitin sulfate (CS) (Aldrich, China), 2,2´-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) (Aldrich, USA), uric acid (UA) (Aldrich, USA), ascorbic acid (AA) (Productos Químicos Monterrey, Mexico) and dopamine (DA) (Aldrich, Germany) were reagent grade and were used without further purification.

2.2 Synthesis of PPy

In a typical synthesis, pyrrole (4 mmol) and the corresponding amount of redox mediator were dissolved in 30 mL of citrate buffer solution (100 mM, pH 4) in a three-necked flask reactor under nitrogen atmosphere. When HA or CS were used, these polymers were also dissolved in the buffer solution.

Next, 2 mL of SBP solution in deionized water were added to the reactor. The polymerization started by the addition of 90.7 μL aliquots of 5% H_2O_2 solution under nitrogen atmosphere at intervals of 5 min during 2.5 h until a total amount of 4 mmol of H_2O_2 was added. The temperature was controlled at 25°C unless otherwise specified.

After the peroxide addition, the mixture was allowed to react for further 2 h and then, the obtained product was collected by filtration, washed with deionized water and vacuum dried at room temperature.

In the cases of using HA or CS, the product was rinsed with deionized water by successive centrifugation cycles at 9,000 rpm for 1 h in order to remove the soluble template and dried by lyophilization.

2.3 Characterizations

The electrical conductivity was measured by the standard two-point method on pellets compressed with a hydraulic press. The measurements were done at room temperature using an Agilent 34410A multimeter (Malaysia). The electrical conductivity reported is the average of three measurements of different pellet samples. X-ray photoelectron spectroscopy (XPS) characterization was carried out on VG-Microtech Mutilab 3000 photoelectron spectrometer equipped with an hemispherical electron analyzer (50 eV pass energy) with 9 channeltrons and a X-ray tube operating at 300 W using the Mg-α line at 1253.6 eV. Fourier transformed infrared spectroscopy (FTIR) spectra were recorded in a Perkin-Elmer Frontier spectrometer (United Kingdom) by the KBr pellet technique. The morphology of samples was studied by using a field-emission scanning electron microscope (FE-SEM), ZEISS Merlin VP Compact (Germany) and a transmission electron microscope (TEM), JEOL 2010F (Japan). Samples were prepared by drying a droplet of the purified aqueous dispersion on an observation grid.

2.4 Electrochemical detection of uric acid

Electrochemical response of PPy as sensor for uric acid was studied with a bipotentiostat microStat 400 from Dropsens (Spain), using carbon nanotube (CNT) screen-printed electrodes 110CNT (Spain). The electrode strips consist of a working electrode (4 mm diameter) of carbon/carboxyl functionalized multi-walled carbon nanotubes, a carbon counter electrode, and a silver pseudo-reference electrode.

Electrode modification with PPy was achieved by casting a drop of PPy suspension. After PPy film deposition, the screen printed electrode was immersed into 9 mL of sodium phosphate buffer solution (PB, 100 mM, pH 7) and placed into a methacrylate cell adapted for screen-printed electrodes. Cyclic voltammetry (CV) experiments were performed at a scanning rate of 50 mV s^{-1}. During the chronoamperometric measurements, aliquots of UA solution were sequentially added under stirring. All the electrochemical measurements were done at room temperature.

3. Results and discussion

3.1 Polymerization yield and electrical conductivity

In order to study the effect of reaction conditions, we carried out several polymerizations summarized in Table 1. Yield and electrical conductivity were used to analyze the best conditions of synthesis for further characterization.

Table 1. Mass yield and conductivity results of PPy as function of initial composition conditions in the synthesis (pyrrole:H_2O_2 molar ratio of 1:1 for all runs).

Run	Sample [a]	Enzyme (KU)	Redox mediator (mmol)	Dopant (mmol) [b]	Mass yield (mg)	Electrical conductivity (S cm^{-1})
1	Py	0	0	0	0	-
2	Py-SBP_d	6 [c]	0	0	0	-
3	Py-SBP	6	0	0	33.0	2.6×10^{-7}
4	Py-SBP-SA	6	0.2	0	274.4	1.2×10^{-8}
5	Py-SBP-AS	6	0.2	0	74.3	2.2×10^{-8}
6	Py-SBP-VA	6	0.2	0	90.1	1.2×10^{-6}
7	Py-SBP-ABTS	6	0.2	0	170.5	1.6×10^{-3}
8	Py-SBP-VA_+	6	0.4	0	118.1	2.1×10^{-6}
9	Py-SBP-VA_+-HA	6	0.4	2	30	$< 6.7 \times 10^{-11}$

10	Py-SBP-VA$_+$-CS	6	0.4	2	247.6	6.1 x 10^{-5}
11	Py-SBP-VA$_+$-CS$_+$	6	0.4	4	369.2	1.5 x 10^{-5}
12	Py-SBP$_+$-VA$_+$-CS$_+$	10	0.4	4	501.6	3.3 x 10^{-5}
13	Py-SBP$_+$-VA$_+$-CS$_+$ [d]	10	0.4	4	447.4	1.1 x 10^{-4}

[a] Samples were identified based on the chemicals initially present in the reaction.

[b] Based on the molecular repeat unit.

[c] Enzyme kept at 100°C for 20 min in aqueous solution.

[d] Synthesis performed at 5°C.

The oxidative polymerization of Py with H_2O_2 in the absence of enzyme did not proceed at pH 4 buffered medium (run 1 in Table 1). The high oxidation potential of H_2O_2 indicates that is thermodynamically capable of oxidize Py but the kinetic limitations disfavor this reaction [25]. Similar result was found when denatured SBP was used (run 2 in Table 1), indicating that iron atoms derived from ferric *heme* group of the enzyme can not catalyze the reaction by themselves.

On the other hand, native SBP led to PPy formation (run 3 in Table 1). This result is in agreement with a previous report that has shown the capability of SBP to oxidize Py monomer even without the aid of redox mediators in a truly enzymatic polymerization [14]. It has been accepted that the catalytic cycle of SBP involves the oxidation of the enzyme active site (from Fe^{3+} to Fe^{4+}) by the H_2O_2 and the subsequent two step reductions to its native state upon oxidation of two Py monomers [14]. The low yield of PPy in this reaction, 12.3% based on monomer, and the low conductivity can be related to the absence of anionic species required for stabilizing the cationic charge of Py moieties.

The addition of natural redox mediators had a positive effect on the reaction yields (run 4-6 in Table 1). SA, AS and VA are aromatic compounds with electron-donating substituents at the benzene ring which decrease the electrochemical potential of these mediators. The efficiency of these compounds as mediators in the oxidation processes has been related to their easy oxidation by redox enzymes, and the stability of their phenoxyl radicals [16]. When SA, AS and VA were added to the enzyme-mediated polymerization of Py, the mass yield increased by 8.3-, 2.2- and 2.7-fold, respectively, as compared to the same reaction carried out without redox mediators (run 3 in Table 1). This means that active species from

the three-redox mediators were able to oxidize Py monomers, accelerating the polymerization kinetics. The efficiency of the mediator depends on several parameters, such as the stability of its oxidized form, its own redox potential, the redox potential of the substrate and steric issues. Interestingly, the enhancement of the polymerization yield was significantly higher for SA than for AS and VA mediators, which evidenced the highest relative ability of the SA to be oxidized by the enzyme and/or to oxidize the Py. This is supported by the cyclic voltammograms of the mediators (supporting information) in the electrolytic solution used as reaction media, that showed lower anodic peaks for SA (0.04 V, 0.26 V, 0.45 V) as compared with AS (0.41 V, 0.66 V) and VA (0.42 V, 0.65 V), which confirmed the highest suitability of this mediator to be oxidized. However, the use of SA did not increase the electrical conductivity of the resultant PPy contrary to that observed when VA was used. This contrasting effect of the SA addition could be attributed to an overoxidation of PPy backbone or some undesired side reactions as chain branching.

The incorporation of ABTS (run 7 in Table 1) produced a 5.2-fold increase of mass yield comparing with the reaction in absence of redox mediator (run 3 in Table 1). This was an expected behavior since previous reports have demonstrated the effectiveness of ABTS as redox mediators in laccase- and HRP-mediated polymerization of pyrrole [11,13]. Moreover, conversely to the effect of SA, AS and also VA, the use of ABTS produced a four-order increase of conductivity of PPy. Under the polymerization conditions, the ABTS sulfonate groups (pKa = 2.2) [26] were fully deprotonated and the mediator existed as a dianion, which could have led to its incorporation as dopant, thereby significantly increasing the conductivity. On the other hand, non-charged SA, AS and VA mediators cannot assume the role as dopants, because their pK_a values are 7.0 [27], 7.8 [27] and 7.9 [28], respectively.

Interestingly, by increasing the concentration of VA (run 8 in Table 1) a 1.3-fold increase of mass yield with respect to the product from lower VA concentration (run 6) was achieved. However, the conductivity was almost insensitive to the increasing of VA amount, indicating a non-significant influence of the redox mediator on the electronic structure of PPy.

In order to improve the electrical properties of synthesized PPy, HA and CS were incorporated to the reaction. Because of the low solubility of HA under buffered conditions,

polymerization was performed in 200 mL of buffer (run 9 in Table 1). Even diluted conditions, the reaction mixture had high viscosity due to the large molecular mass and hydrophilicity of HA. Indeed, a viscous dark mixture was obtained at the end of reaction time. Poor reaction yield and suppressed conductivity evidenced that HA did not promote the biocatalytic polymerization of Py. These results agree with previous findings by Collier and *et*., who reported slow kinetics and suppressed conductivity for single-layer films of PPy/HA obtained from electrochemical method [29].

On the contrary, the addition of CS produced a 2.1-fold increase of mass yield (run 10 in Table 1) as compared with similar synthesis without CS (run 8 in Table 1). The conductivity also increased in one order of magnitude. Unlike HA, which is a relative weak polyanion, it is highly likely that CS was incorporated to the final product as dopant. Comparing both polysaccharides, CS has a relatively higher charge density than HA due to the sulfonic groups, which could explain its better behavior as charge-balancing dopant. We were able to increase the mass yield by increasing the CS in the reaction media, with only a slight decrease in conductivity (run 11 in Table 1). Since the polyanion does not display any intrinsic conductivity, we believe that the electrical conductivity reduction is associated with the increase of the non-conducting phase in the PPy/CS final product. Interestingly, increasing the SBP concentration, higher PPy amount was obtained for same CS concentration, leading to a slight increase of conductivity (run 12 in Table 1). Finally, the polymerization performed at 5°C resulted in one order of magnitude enhancement of conductivity (run 13 in Table 1) as compared with similar procedure at 25°C (run 12 in Table 1), probably by decreasing the nucleophilic reactions of the Py radical with water. This result agrees with previous reports that indicated higher conductivities for PPy synthesized at lower temperatures via enzymatic [14], electrochemical [30] and chemical procedures [31].

3.2 Physicochemical and morphological characterizations

In order to analyze the effect of the ABTS and VA/CS on the chemical composition, we compared these samples by XPS analysis and the semiquantitative surface chemical composition results are shown in Table 2.

The high content of oxygen in the sample Py-SBP suggests that overoxidation of the polymer took place, or that the enzyme was entangled in the obtained polymer, probably linked to the PPy by physical interactions. In the sample Py-SBP-ABTS, the sulfur content confirmed the presence of ABTS as dopant. In fact, the N:S atomic ratio indicated that at least, at surface level, an excess of the ABTS amount required to achieve the maximum theoretical doping of PPy remained in the sample. This might be considered inconvenient for some biomedical application since ABTS toxicity has not been well examined, but its oxidizing nature suggests that it might behave as an oxidative stress inductor for cells. The presence of sulfur in sample Py-SBP-VA_{+}-CS_{+} also corroborated the CS incorporation.

Table 2. XPS data of PPy synthesized in the presence of the SBP, SBP/ABTS and SBP/VA/CS.

	Atomic composition (%)				Atomic ratio N:S
	C	N	S	O	
Py-SBP	69.2	12.7	-	18.1	-
Py-SBP-ABTS	70.5	12.3	5.0	12.2	2.5
Py-SBP-VA_{+}-CS_{+}	69.8	9.3	1.9	18.4	4.9

The C1s and N1s core-level spectra are shown in Figure 1. For all samples, the C1s peak was deconvoluted into three components. The main component at 284.5 eV arises from the C-C bonds. The peak at 286.1 eV can be related to C-N/C-O moieties whereas the contribution at 287.9 eV was derived from C=O bond [13,32,33]. Signals of carbon bounded to oxygen are typically found in PPy samples synthesized either by enzymatic [13], electrochemical [32] or chemical methods [33]. ABTS or VA/CS incorporation diminished the signal intensity of C=O bond (5.9 and 7.8%, respectively) as compared with sample Py-SBP (12.4%). This implies that ABTS- or CS-PPy association could hinder in some extent the negative effects of overoxidation of the conducting polymer. Interestingly, the highest amount of C-O contribution was found in sample Py-SBP-VA_{+}-CS_{+}, which agrees with the rich presence of this bond in CS, suggesting it was successfully incorporated in the polymer.

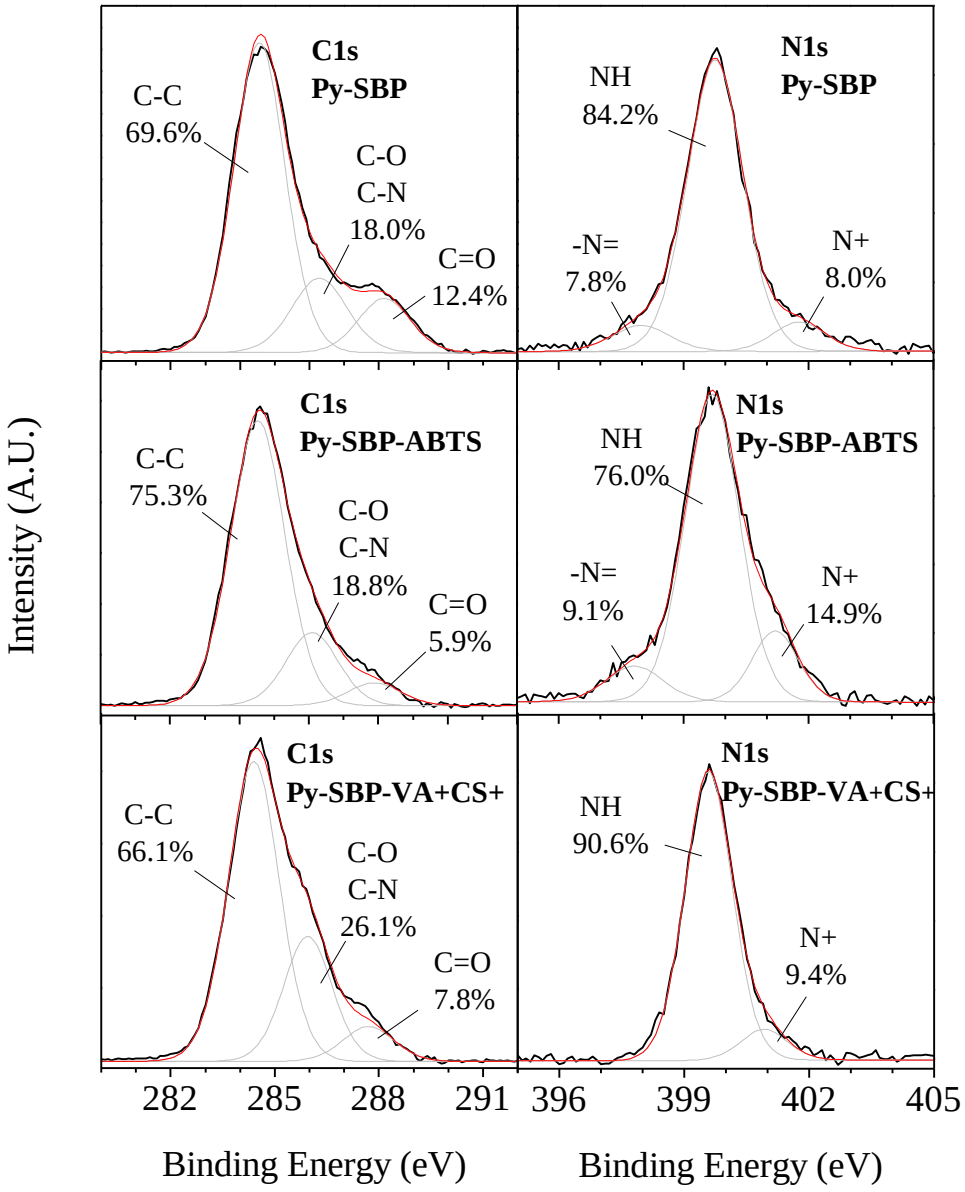


Figure 1. C1s and N1s core-level spectra of samples Py-SBP, Py-SBP-ABTS and Py-SBP-VA_{+}-CS_{+}.

The XPS spectra of N 1s region are also included in Figure 1. Samples Py-SBP and Py-SBP-ABTS displayed the lowest binding energy component at 397.9 eV that was attributed to structural defects in the form of imine nitrogen (-N=), followed by the signal of neutral amine nitrogen (-NH-) at 399.7 eV with the strongest intensity and a peak above 400 eV attributed to positively charged nitrogen atoms [34]. The relative content of charged

nitrogen correlates with conductivity measured in samples (Table1). The sample Py-SBP-VA_+-CS_+ showed only the two components of higher binding energy. The absence of imine-type nitrogen suggests that CS promoted the orderly growth of PPy backbone, preventing the formation of structural defects. Since imine defects interrupt conjugation, it would be expected that a lower concentration of such defects would produce an extended conjugation and high values of conductivity. So, the relative poor conductivity obtained in Py-SBP-VA_+-CS_+ compared with Py-SBP-ABTS and some other samples reported in literature [13,14], can be attributed to steric restrictions of the bulky CS dopant which could limit the interchain/intergrain hopping of charge carriers. Similar observations have been reported about the effects of bulky dopants on electrical conductivity [35]. However, this peak is higher for the Py-SBP-ABTS sample, and it could be related with the presence of ABTS in the polymer.

Figure 2 shows the FTIR spectra of (a) Py-SBP, (b) Py-SBP-ABTS and (c) Py-SBP-VA_+-CS_+ samples. The position of the typical bands of PPy is quite similar in all spectra. The broad band located from 3600 to 3000 cm^{-1} was attributed to stretching mode of N-H vibration. The band at 1578-1562 cm^{-1} was assigned to the C-C stretching vibration of Py ring. Signals at 1479 and 1484 cm^{-1} were detected for Py-SBP-ABTS and Py-SBP-VA_+-CS_+, respectively, which correspond to C-N stretching vibration in the ring. Band at 1219-1200 cm^{-1} was attributed to the breathing vibration of the Py ring. Sharp band at 1049-1041 cm^{-1} was correlated to the C-H and N-H in plane deformation vibration. The strong signal at 932-924 cm^{-1} was related to C-H out of plane deformation. Band at 804-788 cm^{-1} was attributed to C-H out of plane ring deformation [36]. The spectra of all samples contain a signal at 1711-1682 cm^{-1}, which can be assigned to carbonyl vibrations. This feature agrees with the presence of C=O signal in XPS spectra for same samples (Figure 1).

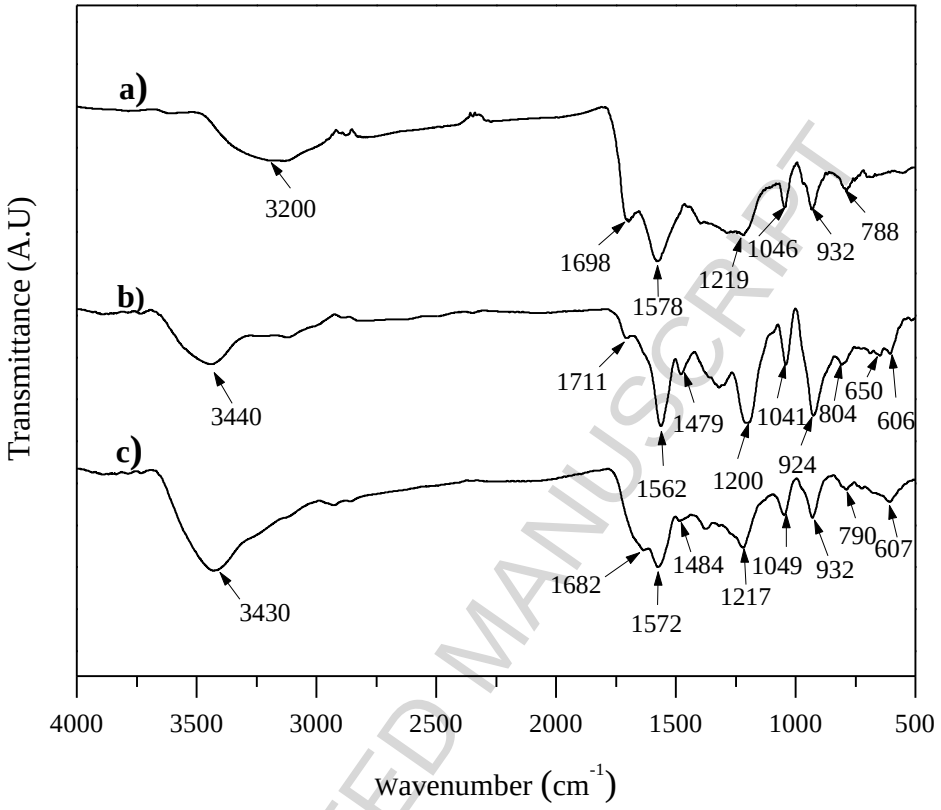


Figure 2. FTIR spectra of (a) Py-SBP, (b) Py-SBP-ABTS and (c) Py-SBP-VA_+-CS_+ samples.

The spectrum of PPy synthesized in the presence of ABTS shows additional bands at 650 and 606 cm^{-1} that correspond to the bending modes of the sulfonate groups on ABTS. The characteristic bands of sulfonate groups were also detected around 607 cm^{-1} for sample Py-SBP-VA_+-CS_+ [11,12].

Figure 3 illustrates the FE-SEM micrographs of (a) Py-SBP, (b) Py-SBP-ABTS and (c) Py-SBP-VA_+-CS_+ samples. The powdery samples consisted of globular-shaped agglomerates with maximum sizes around 200 nm. This shape is typical of conducting polymers, whose primary particles have an intrinsic tendency to agglomerate due to their high surface tension arising from electrostatic and van der Waals interactions [37,38]. It should also be noticed that the average dimension of these clusters seems to decrease in the order Py-SBP > Py-SBP-ABTS > Py-SBP-VA_+-CS_+.

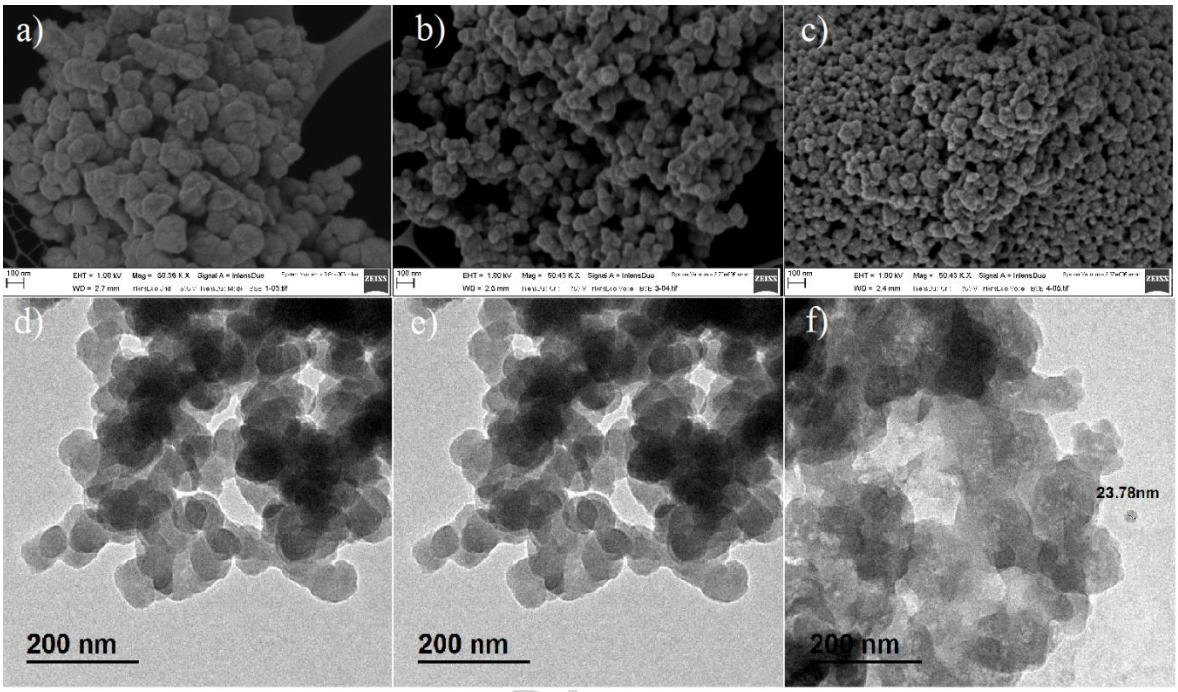


Figure 3. FE-SEM images of (a) Py-SBP, (b) Py-SBP-ABTS and (c) Py-SBP-VA_+-CS_+ samples with scale bar of 100 nm. TEM images of (d) Py-SBP, (e) Py-SBP-ABTS and (f) Py-SBP-VA_+-CS_+ samples.

TEM micrographs of the samples Py-SBP, Py-SBP-ABTS and Py-SBP-VA_+-CS_+ are also included in Figure 3 d, e and f, respectively. Detailed observations of these images revealed that the agglomerate structures are formed by quasi-spherical nanoparticles. Other authors have found similar structures to those reported here for PPy prepared by H_2O_2-mediated reactions. For example, Leonavicius *et al.* obtained micrometric arrangements of 30 nm-sized spherical PPy particles in H_2O_2 initiated polymerization performed in the presence of sodium dodecyl sulfate [38].

Isolated particles of sample Py-SBP-VA_+-CS_+ showed dimensions of around 25 nm (Figure 3e). The nanometric size of PPy synthesized in the presence of natural precursors can be considered an additional advantage for its biomedical applications since it will allow optimizing the interaction of the polymer with biological environments.

3.3 Determination of uric acid

In order to evaluated the electroactivity of PPy synthesized in the presence of natural precursors, we performed the electrochemical detection of uric acid (UA) using CNT screen-printed electrodes modified with sample Py-SBP-VA_{+}-CS_{+}. UA is the primary product of purine metabolism in the human body. It has been shown that abnormal UA levels can be associated with diseases such as gout, hyperuricaemia and Lesch-Nyham syndrome [39].

Figure 4a shows cyclic voltammograms of unmodified and PPy-modified CNT electrodes in PB pH 7. In the absence of analyte, both electrodes exhibited no electrochemical response in buffer media. The addition of 80 μM UA (Figure 4b) produced an increase of oxidation current with a peak recorded at 0.32-0.35 V corresponding to the oxidation of UA as it has been reported elsewhere [40,41]. It can be clearly observed that the peak current magnitude of PPy-modified CNT electrode was about three times higher than that recorded in unmodified CNT electrode. This enhancement of the amperometric sensitivity evidenced the electrocatalytic activity of modified electrode toward UA oxidation, and ultimately demonstrated the suitability of Py-SBP-VA_{+}-CS_{+} sample as mediator to shuttle the electrons between the analyte and the working electrode.

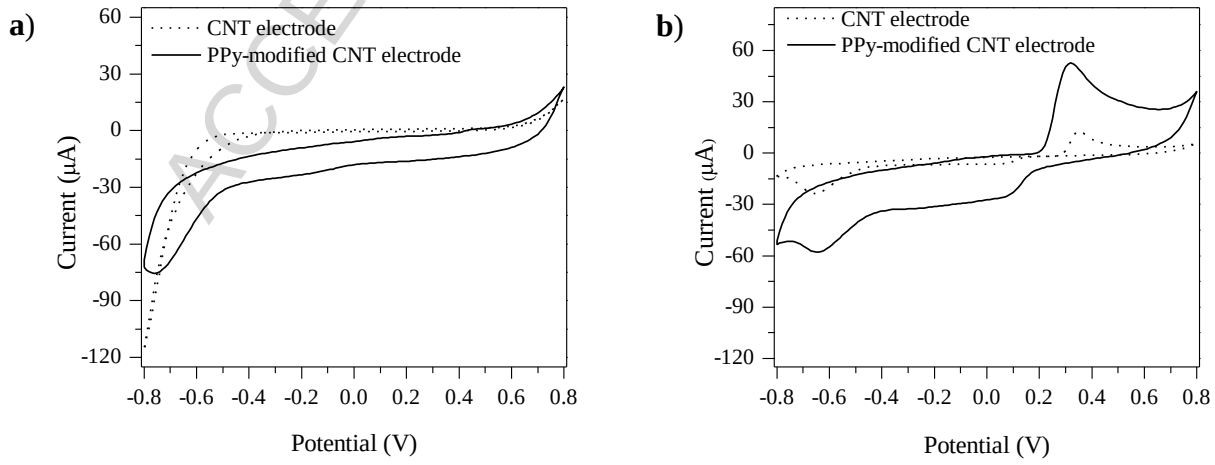


Figure 4. Cyclic voltammograms of unmodified and PPy-modified CNT electrodes in the (a) absence and (b) presence of 80 μM UA in PB pH 7 at scan rate of 50 mV s^{-1}.

Figure 5 illustrates the amperometric response of CNT screen-printed electrodes modified with sample Py-SBP-VA_+-CS_+ under successive additions of UA. Inset shows the corresponding calibration curve of steady-state currents versus accumulated concentration of analyte. The currents increased linearly with UA concentration between 5 and 97 μM. This response indicated a stable and efficient catalytic properties of sample Py-SBP-VA_+-CS_+. The sensitivity against UA in the linear segment was found to be 47 μA mM^{-1}, which is comparable with values reported previously for non-enzymatic UA sensors [42].

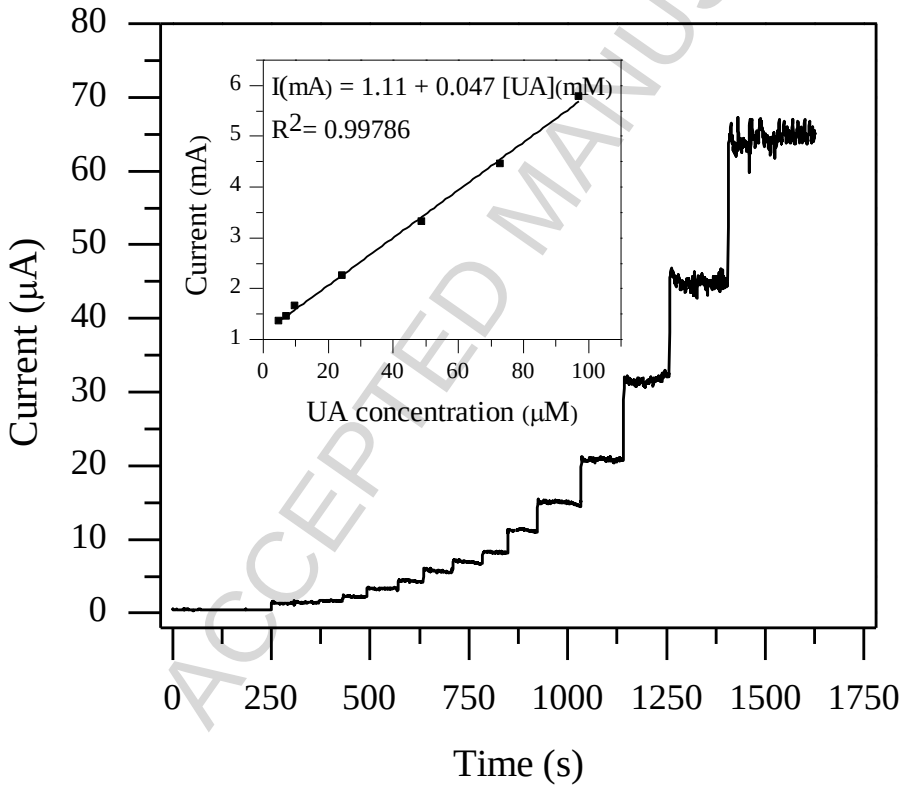


Figure 5. Amperometric response of CNT screen-printed electrode modified with sample Py-SBP-VA_+-CS_+ upon the successive addition of UA in PB pH 7, with an applied potential +0.36 V *vs* Ag (Inset: plot of steady-state currents versus UA concentration).

The selectivity of the PPy-modified CNT electrode toward UA oxidation was evaluated by sequential additions of dopamine (DA) and ascorbic acid (AA). UA, AA and DA are electroactive constituents of body fluids and they have similar oxidation potentials at carbon-based electrodes [43,44]. Figure 6 shows the chronoamperometric response of PPy-

modified CNT electrode upon sequential additions of 10 μmoles of UA, 20 μmoles of UA, 20 μmoles of DA, 20 μmoles of AA, 20 μmoles of UA and 20 μmoles of UA. As it can be seen in the plot, the DA addition produced a current increase of around 60% of the current caused by previous UA aliquot. On the other hand, negligible current response was found toward AA, as indicative of minimal interference in this case.

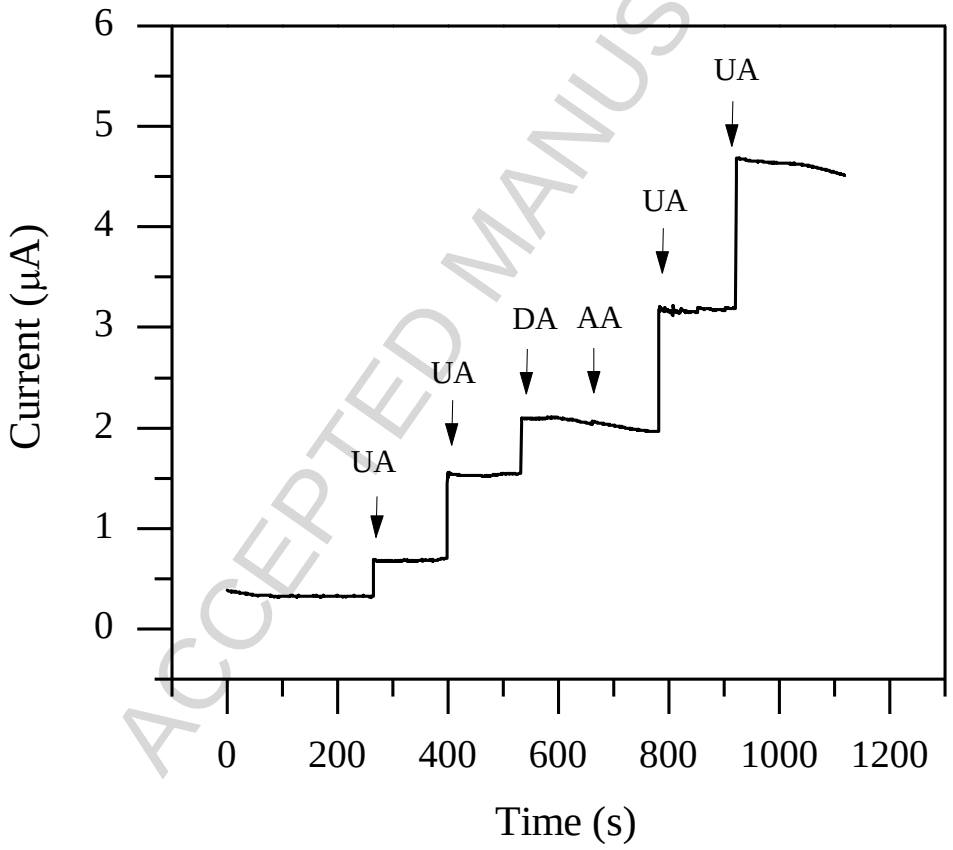


Figure 6. Amperometric response of CNT screen-printed electrode modified with sample Py-SBP-VA+-CS + upon the successive addition of 10 μmoles of UA, 20 μmoles of UA, 20 μmoles of DA, 20 μmoles of AA, 20 μmoles of UA and 20 μmoles of UA to 8 mL of PB pH 7, with an applied potential +0.36 V *vs* Ag.

4. Conclusions

A new synthetic route was developed for the preparation of polypyrrole via enzyme-mediated polymerization using redox mediators of natural origin and soybean peroxidase as catalyst. All three redox mediators, acetosyringone, syringaldehyde and vanillin, increased the reaction yield with non-significant influence on the electronic structure of the resultant polypyrrole. Natural redox mediators might have advantages over ABTS for biomedical applications such as *in-vivo* sensors. Enhancement of electrical conductivity of polymer was achieved by incorporation of chondroitin sulfate as charge-balancing dopant. It was also shown by XPS analysis that the polysaccharide promoted the orderly growth of polypyrrole backbone, preventing the formation of structural defects. Polypyrrole synthesized in the presence of vanillin and chondroitin sulfate was used as sensor in the electrochemical detection of uric acid, owing to its electrocatalytic activity toward uric acid oxidation and the linear amperometric response to the analyte concentration. Hybrid system of polypyrrole-chondroitin sulfate combines the electrical properties of the polymer with the biofunctionality and innocuousness of the polysaccharide, therefore it can be considered as a potential platform for biomedical applications.

Acknowledgements

This work was supported by the Consejo Nacional de Ciencia y Tecnología (CONACYT), Mexico (Grant Ciencia Básica 2012-N°180280). Financial support from the Spanish Ministerio de Economía y Competitividad and FEDER funds (MAT2013-42007-P) and from the Generalitat Valenciana (PROMETEO2013/038) is also gratefully acknowledged. Génesis Adilene Grijalva Bustamante acknowledges CONACYT for the scholarship during this study.

References

[1] J. Pelto, S. Haimi, E. Puukilainen, P.G. Whitten, G.M. Spinks, M. Bahrami-Samani, et al., Electroactivity and biocompatibility of polypyrrole-hyaluronic acid multi-

walled carbon nanotube composite., J. Biomed. Mater. Res. A. 93 (2010) 1056–67.

[2] D.. Ateh, H.. Navsaria, P. Vadgama, Polypyrrole-based conducting polymers and interactions with biological tissues, J. R. Soc. Interface. 3 (2006) 741–752.

[3] L. Ghasemi-Mobarakeh, M.P. Prabhakaran, M. Morshed, M.H. Nasr-Esfahani, H. Baharvand, S. Kiani, et al., Application of conductive polymers, scaffolds and electrical stimulation for nerve tissue engineering., J. Tissue Eng. Regen. Med. 5 (2011) e17–35.

[4] C. Saravanan, R.C. Shekhar, S. Palaniappan, Synthesis of Polypyrrole Using Benzoyl Peroxide as a Novel Oxidizing Agent, Macromol. Chem. Phys. 207 (2006) 342–348.

[5] C.F. Hsu, L. Zhang, H. Peng, J. Travas-Sejdic, P.A. Kilmartin, Scavenging of DPPH free radicals by polypyrrole powders of varying levels of overoxidation and/or reduction, Synth. Met. 158 (2008) 946–952.

[6] J.M. Pringle, J. Efthimiadis, P.C. Howlett, J. Efthimiadis, D.R. MacFarlane, A.B. Chaplin, et al., Electrochemical synthesis of polypyrrole in ionic liquids, Polymer 45 (2004) 1447–1453.

[7] Z. Zhou, W. Zhu, J. Liao, S. Huang, J. Chen, T. He, et al., Chondroitin sulphate-guided construction of polypyrrole nanoarchitectures., Mater. Sci. Eng. C. Mater. Biol. Appl. 48 (2015) 172–8.

[8] W. Liu, J. Kumar, S. Tripathy, K.J. Senecal, L. Samuelson, Enzymatically Synthesized Conducting Polyaniline, J. Am. Chem. Soc. 121 (1999) 71–78.

[9] R. Cruz-Silva, J. Romero-García, J.L. Angulo-Sánchez, A. Ledezma-Pérez, E. Arias-Marín, I. Moggio, et al., Template-free enzymatic synthesis of electrically conducting polyaniline using soybean peroxidase, Eur. Polym. J. 41 (2005) 1129–1135.

[10] A. Ramanavicius, A. Kausaite, A. Ramanaviciene, J. Acaite, A. Malinauskas, Redox enzyme – glucose oxidase – initiated synthesis of polypyrrole, Synth. Met. 156 (2006) 409–413.

[11] H.-K. Song, G.T.R. Palmore, Conductive polypyrrole via enzyme catalysis., J. Phys. Chem. B. 109 (2005) 19278–87.

[12] Y.N. Kupriyanovich, B.G. Sukhov, S.A. Medvedeva, A.I. Mikhaleva, T.I.

Vakul'skaya, G.F. Myachina, et al., Peroxidase-catalysed synthesis of electroconductive polypyrrole, Mendeleev Commun. 18 (2008) 56–58.

[13] R. Cruz-Silva, E. Amaro, A. Escamilla, M.E. Nicho, S. Sepulveda-Guzman, L. Arizmendi, et al., Biocatalytic synthesis of polypyrrole powder, colloids, and films using horseradish peroxidase., J. Colloid Interface Sci. 328 (2008) 263–9.

[14] R. Bouldin, S. Ravichandran, A. Kokil, R. Garhwal, S. Nagarajan, J. Kumar, et al., Synthesis of polypyrrole with fewer structural defects using enzyme catalysis, Synth. Met. 161 (2011) 1611–1617.

[15] K. Junker, G. Zandomeneghi, L.D. Schuler, R. Kissner, P. Walde, Enzymatic polymerization of pyrrole with Trametes versicolor laccase and dioxygen in the presence of vesicles formed from AOT (sodium bis-(2-ethylhexyl) sulfosuccinate) as templates, Synth. Met. 200 (2015) 123–134.

[16] A.I. Cañas, S. Camarero, Laccases and their natural mediators: biotechnological tools for sustainable eco-friendly processes., Biotechnol. Adv. 28 (2010) 694–705.

[17] R.A.A. Muzzarelli, F. Greco, A. Busilacchi, V. Sollazzo, A. Gigante, Chitosan, hyaluronan and chondroitin sulfate in tissue engineering for cartilage regeneration: a review., Carbohydr. Polym. 89 (2012) 723–39.

[18] N. German, A. Kausaite-Minkstimiene, A. Ramanavicius, T. Semashko, R. Mikhailova, A. Ramanaviciene, The use of different glucose oxidases for the development of an amperometric reagentless glucose biosensor based on gold nanoparticles covered by polypyrrole, Electrochim. Acta. 169 (2015) 326–333.

[19] H. Beitollahi, M. Mostafavi, Nanostructured Base Electrochemical Sensor for Simultaneous Quantification and Voltammetric Studies of Levodopa and Carbidopa in Pharmaceutical Products and Biological Samples, Electroanalysis 26 (2014) 1090–1098.

[20] H. Beitollahi, I. Sheikhshoaie, Selective voltammetric determination of norepinephrine in the presence of acetaminophen and folic acid at a modified carbon nanotube paste electrode, J. Electroanal. Chem. 661 (2011) 336–342.

[21] E. Molaakbari, A. Mostafavi, H. Beitollahi, R. Alizadeh, Synthesis of ZnO nanorods and their application in the construction of a nanostructure-based electrochemical sensor for determination of levodopa in the presence of carbidopa., Analyst 139

(2014) 4356–64.

[22] A. Henriksen, O. Mirza, C. Indiani, K. Teilum, G. Smulevich, K.G. Welinder, et al., Structure of soybean seed coat peroxidase: a plant peroxidase with unusual stability and haem-apoprotein interactions., Protein Sci. 10 (2001) 108–15.

[23] J.P. McEldoon, J.S. Dordick, Unusual Thermal Stability of Soybean Peroxidase, Biotechnol. Prog. 12 (1996) 555–558.

[24] H. Wright, J.A. Nicell, Characterization of soybean peroxidase for the treatment of aqueous phenols, Bioresour. Technol. 70 (1999) 69–79.

[25] H.V.R. Dias, M. Fianchini, R.M.G. Rajapakse, Greener method for high-quality polypyrrole, Polymer 47 (2006) 7349–7354.

[26] S.L. Scott, W.J. Chen, A. Bakac, J.H. Espenson, Spectroscopic parameters, electrode potentials, acid ionization constants, and electron exchange rates of the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) radicals and ions, J. Phys. Chem. 97 (1993) 6710–6714.

[27] F. Xu, Effects of redox potential and hydroxide inhibition on the pH activity profile of fungal laccases, J. Biol. Chem. 272 (1997) 924–928.

[28] R.J. Prankerd, Profiles of Drug Substances, Excipients and Related Methodology, first ed., Academic Press, London, 2007.

[29] J.H. Collier, J.P. Camp, T.W. Hudson, C.E. Schmidt, Synthesis and characterization of polypyrrole-hyaluronic acid composite biomaterials for tissue engineering applications., J. Biomed. Mater. Res. 50 (2000) 574–84.

[30] A. Kassim, Z.B. Basar, H.N.M.E. Mahmud, Effects of preparation temperature on the conductivity of polypyrrole conducting polymer, J. Chem. Sci. 114 (2002) 155–162.

[31] W. Liang, J. Lei, C.R. Martin, Effect of synthesis temperature on the structure, doping level and charge-transport properties of polypyrrole, Synth. Met. 52 (1992) 227–239.

[32] W. Prissanaroon, N. Brack, P.J. Pigram, J. Liesegang, T.J. Cardwell, Surface and electrochemical study of DBSA-doped polypyrrole films grown on stainless steel, Surf. Interface Anal. 33 (2002) 653–662.

[33] L. Ruangchuay, J. Schwank, A. Sirivat, Surface degradation of α-naphthalene

sulfonate-doped polypyrrole during XPS characterization, Appl. Surf. Sci. 199 (2002) 128–137.

[34] L.E. Valencia Castro, C.J. Pérez Martínez, T. del Castillo Castro, M.M. Castillo Ortega, J.C. Encinas, Chemical polymerization of pyrrole in the presence of l -serine or l -glutamic acid: Electrically controlled amoxicillin release from composite hydrogel, J. Appl. Polym. Sci. 132 (2015) 41804, doi: 10.1002/app.41804.

[35] A. Kaynak, R. Beltran, Effect of synthesis parameters on the electrical conductivity of polypyrrole-coated poly(ethylene terephthalate) fabrics, Polym. Int. 52 (2003) 1021–1026.

[36] M. Omastová, M. Trchová, J. Kovářová, J. Stejskal, Synthesis and structural study of polypyrroles prepared in the presence of surfactants, Synth. Met. 138 (2003) 447–455.

[37] U.M. Casado, M.I. Aranguren, N.E. Marcovich, Preparation and characterization of conductive nanostructured particles based on polyaniline and cellulose nanofibers., Ultrason. Sonochem. 21 (2014) 1641–8.

[38] K. Leonavicius, A. Ramanaviciene, A. Ramanavicius, Polymerization model for hydrogen peroxide initiated synthesis of polypyrrole nanoparticles., Langmuir. 27 (2011) 10970–6.

[39] M. Ates, A.S. Sarac, Conducting polymer coated carbon surfaces and biosensor applications, Prog. Org. Coatings. 66 (2009) 337–358.

[40] L. Wang, P. Huang, J. Bai, H. Wang, Voltammetric sensing of uric acid and ascorbic acid with poly (p-toluene sulfonic acid) modified electrode, Int. J. Electrochem. Sci. 1 (2006) 334–342.

[41] S. Shahrokhian, M. Azimzadeh, M.K. Amini, Modification of glassy carbon electrode with a bilayer of multiwalled carbon nanotube/tiron-doped polypyrrole: Application to sensitive voltammetric determination of acyclovir., Mater. Sci. Eng. C. Mater. Biol. Appl. 53 (2015) 134–41.

[42] D. Oukil, L. Benhaddad, R. Aitout, L. Makhloufi, F. Pillier, B. Saidani, Electrochemical synthesis of polypyrrole films doped by ferrocyanide ions onto iron substrate: Application in the electroanalytical determination of uric acid, Sensors Actuators B Chem. 204 (2014) 203–210.

[43] E. Popa, Y. Kubota, D.A. Tryk, A. Fujishima, Selective Voltammetric and Amperometric Detection of Uric Acid with Oxidized Diamond Film Electrodes, Anal. Chem. 72 (2000) 1724–1727.

[44] J.-M. Zen, P.-J. Chen, A Selective Voltammetric Method for Uric Acid and Dopamine Detection Using Clay-Modified Electrodes, Anal. Chem. 69 (1997) 5087–5093.

Figure Captions

Figure 1. C1s and N1s core-level spectra of samples Py-SBP, Py-SBP-ABTS and Py-SBP-VA_{+}-CS_{+}.

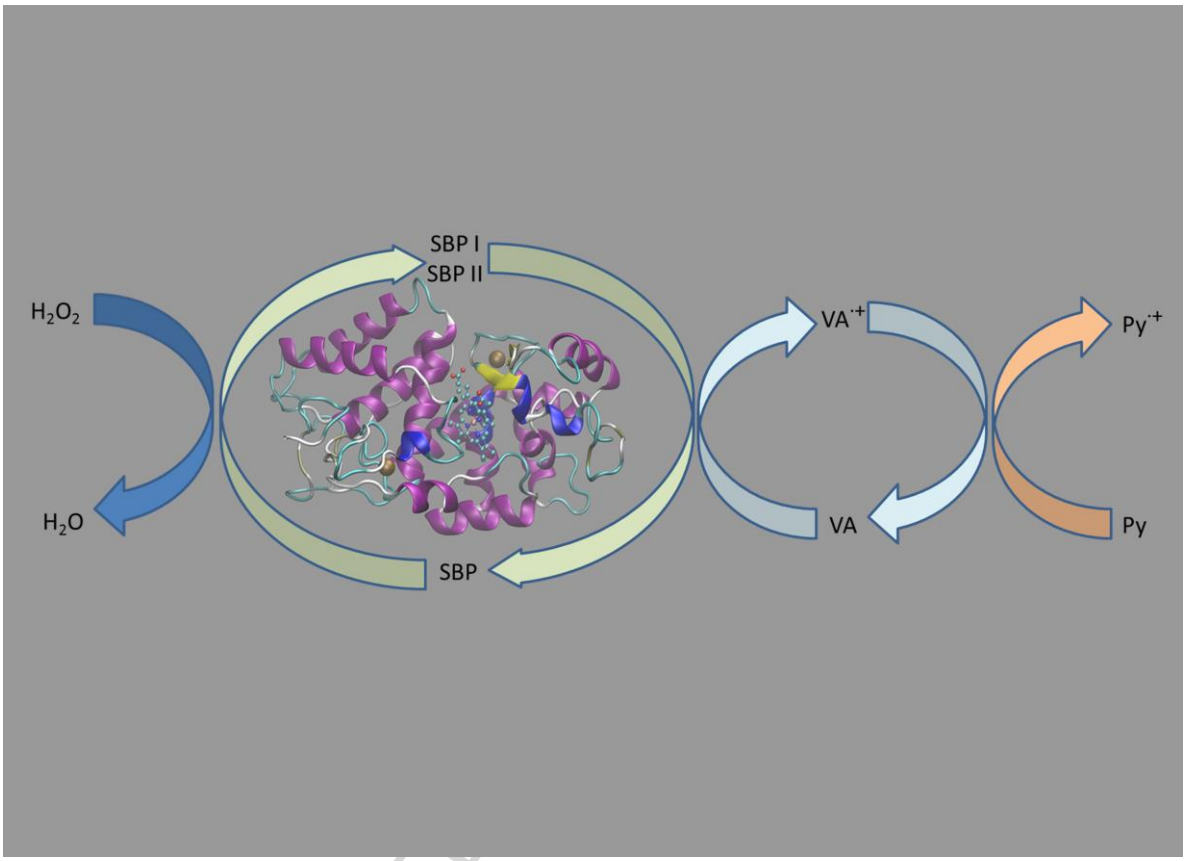
Figure 2. FTIR spectra of (a) Py-SBP, (b) Py-SBP-ABTS and (c) Py-SBP-VA_{+}-CS_{+} samples.

Figure 3. FE-SEM images of (a) Py-SBP, (b) Py-SBP-ABTS and (c) Py-SBP-VA_{+}-CS_{+} samples with scale bar of 100 nm. TEM images of (d) Py-SBP, (e) Py-SBP-ABTS and (f) Py-SBP-VA_{+}-CS_{+} samples.

Figure 4. Cyclic voltammograms of unmodified and PPy-modified CNT electrodes in the (a) absence and (b) presence of 80 μM UA in PB pH 7 at scan rate of 50 mV s^{-1}.

Figure 5. Amperometric response of CNT screen-printed electrode modified with sample Py-SBP-VA_{+}-CS_{+} upon the successive addition of UA in PB pH 7, with an applied potential +0.36 V *vs* Ag (Inset: plot of steady-state currents versus UA concentration).

Figure 6. Amperometric response of CNT screen-printed electrode modified with sample Py-SBP-VA+-CS + upon the successive addition of 10 μmoles of UA, 20 μmoles of UA, 20 μmoles of DA, 20 μmoles of AA, 20 μmoles of UA and 20 μmoles of UA to 8 mL of PB pH 7, with an applied potential +0.36 V *vs* Ag.



Graphical abstract

Highlights

- A new method of pyrrole polymerization using naturally occurring redox mediators and doping agents was studied.
- The catalytic efficiency of different redox mediators towards pyrrole oxidation was evaluated.
- Two different naturally occurring polymers were studied as bifunctional steric stabilizer/doping agents.
- Polypyrrole improves the amperometric response of carbon nanotube screen printed electrodes towards uric acid sensing.